Behavioural Brain Research 325 (2017) 268–277

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

The association of serotonin receptor 3A methylation with maternal violence exposure, neural activity, and child aggression☆

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HIGHLIGHTS

- Maternal severity of interpersonal violence exposure (IPV) was associated with diagnosis of maternal post-traumatic stress disorder (PTSD).
- Maternal IPV-PTSD was in turn associated with disturbed child attachment.
- *HTR3A* gene methylation was linked to maternal IPV exposure and aggressive behavior and disturbed child attachment and self-endangering behavior.
- *HTR3A* methylation at the CpG2_III site was linked to decreased medial prefrontal cortical activity in response to menacing relational stimuli.

ARTICLE INFO

Article history:
Received 29 April 2016
Received in revised form 4 October 2016
Accepted 5 October 2016
Available online 5 October 2016

Keywords:
Maternal posttraumatic stress disorder (PTSD)
Interpersonal violence
Serotonin receptor
Epigenetics
fMRI
Attachment disorder

ABSTRACT

Background: Methylation of the serotonin 3A receptor gene (*HTR3A*) has been linked to child maltreatment and adult psychopathology. The present study examined whether *HTR3A* methylation might be associated with mothers' lifetime exposure to interpersonal violence (IPV), IPV-related psychopathology, child disturbance of attachment, and maternal neural activity.
Methods: Number of maternal lifetime IPV exposures and measures of maternal psychopathology including posttraumatic stress disorder (PTSD), major depression and aggressive behavior (AgB), and a measure of child attachment disturbance known as "secure base distortion" (SBD) were assessed in a sample of 35 mothers and children aged 12–42 months. Brain fMRI activation was assessed in mothers using 30-s silent film excerpts depicting menacing adult male-female interactions versus prosocial and neutral interactions. Group and continuous analyses were performed to test for associations between clinical and fMRI variables with DNA methylation.
Results: Maternal IPV exposure-frequency was associated with maternal PTSD; and maternal IPV-PTSD was in turn associated with child SBD. Methylation status of several CpG sites in the *HTR3A* gene was associated with maternal IPV and IPV-PTSD severity, AgB and child SBD, in particular, self-endangering behavior. Methylation status at a specific CpG site (CpG2_III) was associated with decreased medial prefrontal cortical (mPFC) activity in response to film-stimuli of adult male-female interactions evocative of violence as compared to prosocial and neutral interactions.
Conclusions: Methylation status of the *HTR3A* gene in mothers is linked to maternal IPV-related psychopathology, trauma-induced brain activation patterns, and child attachment disturbance in the form of SBD during a sensitive period in the development of self-regulation.

☆ This paper is designated for the Special Issue on "The development of attachment: integrating genes, brain, behavior, and environment".
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http://dx.doi.org/10.1016/j.bbr.2016.10.009

1. Introduction

1.1. The serotonergic system as linked to early life stress and aggression

Dysfunction of the serotonergic system has been linked both to early life stress and to aggression [1]. And yet the potential role of serotonin in the intergenerational transmission of violence and related trauma remains largely unknown [2]. Recent work indicates that serotonin modulates brain circuits in a cell-type specific manner through a large family of receptors [3]. In addition, serotonin neurons located in the midbrain are molecularly diverse [4] with subsets of serotonin neurons possibly regulating different types of physiological functions [5]. Using recent methods of circuit dissection in rodents, the activity of raphe serotonergic neurons has been shown to control a range of emotional behaviors [6]. More specifically, serotonin has been proposed to modulate aggressive behaviors in animal and human experimental models [7,8]. Whether subsets of serotonin neurons specifically control aggression remains to be determined.

Correlative studies in humans have focused on the primary metabolite of serotonin, 5-hydroxyindoleacetic acid (5-HIAA). Using this measure of serotonin function, low cerebrospinal fluid (CSF) 5-HIAA concentrations have been associated with peer-peer aggression in non-human primates [9]. In addition, low maternal CSF 5-HIAA was associated with maternal abuse of infants among macaque monkeys [10]. In humans, a low concentration of 5-HIAA has been associated with lifetime aggression, impulsive acts of violence and antisocial behavior across 20 separate studies [11]. Finally, variants in serotonin-related genes including the serotonin receptor 2A (*HTR2A*) and the monoamine oxydase A (*MAOA*) have been associated with impulsive aggression towards self and others in a number of human studies [12,13].

Interactions between serotonin-related genetic variants and early life stress including typically traumatogenic events such as child maltreatment have been observed in rodents, macaques and human [14]. However, few studies have examined the link between serotonin-related genetic variants and post-traumatic stress disorder (PTSD). An interaction between 5-HT2A variants and childhood sexual abuse exposure has been shown to increase risk for PTSD and comorbid depression in an African American university-based population [15].

1.2. Epigenetics of the serotonin receptor 3A and early-onset exposure to maltreatment and other forms of interpersonal violence

More recently, epigenetic studies have started to establish a link between early-life adversity and methylation levels in stress-related genes in psychiatric conditions, including borderline personality disorder, generalized anxiety symptoms, and PTSD [16,17]. In addition some of these studies have started to investigate whether methylation changes could be correlated with stress-related patterns of neural activation measured by fMRI [18,19].

In this regard, the serotonin receptor 3A (HTR3A) is of particular interest. In rodents, the HTR3A is specifically expressed in specific subsets of interneurons [20,21]. It controls early cellular processes involved in circuit formation [21,22], regulates neuronal amygdala excitability [23] and is required for fear extinction [24]. In humans, genetic variation in the HTR3A has been shown to interact with early-life adversity [25,26] and has been associated with psychiatric disorders including bipolar disorder [27,28] and PTSD [15]. More recently, childhood maltreatment including physical abuse has been shown to modulate the methylation status of several CpG sites in the promoter regions of the *HTR3A* gene in individuals diagnosed with ADHD, bipolar disorder or borderline personality disorder [29]. Interestingly, the methylation status of a specific CpG site (named CpG2_III based on the Perroud et al. study [29]) was found to be strongly modulated by a functional SNP (rs1062613) located at 1 base-pair away from CpG2_III. Furthermore, both the CpG2_III methylation and rs1062613 were recently found to modulate the binding of the transcription factor CCCTC-binding factor (CTCF) [30], strongly suggesting functional biological significance.

Of note, none of the aforementioned studies looking at methylation of the *HTR3A* gene thus far has examined whether PTSD was present or comorbid with the psychiatric disorders characterizing the sample. In the instance of PTSD being linked to early-onset, repeated and chronic exposure to maltreatment and other forms of interpersonal violence, HPA-axis functioning and related methylation of the glucocorticoid receptor have been found to show patterns that are distinct from those associated with mood disorders, despite frequent comorbidity [31–33]. And therefore, methylation studies of stress-related genes *HTR3A* gene are needed within samples of patients suffering from PTSD related to childhood maltreatment and subsequent exposure to other forms of interpersonal violence.

In the present paper, we therefore examined within a sample of adult women who were mothers of young children (ages 12–42 months), methylation of the maternal *HTR3A* gene promoter region, with specific attention to CpG2_III, and its relationship with maternal life stress, interpersonal violence related post-traumatic stress disorder (IPV-PTSD), aggression and neural activity in response to a trauma trigger. We then also asked if child psychopathology in the form of a characteristic attachment disturbance within this high-risk sample, and in particular child self-endangering behavior within the context of this attachment disturbance, might also be associated with one or more of the maternal variables in question. To our knowledge, this is the first paper to explore these relationships individually and together.

1.3. Hypotheses

Our hypotheses are the following:

Methylation of the maternal *HTR3A* gene promoter region, in particular at CpG2_III, will be associated with the following:

1) Maternal exposure to interpersonal violence since childhood (i.e. physical abuse, domestic violence exposure and subsequent victimization) as well as with related maternal PTSD and aggression
2) Child symptoms such as separation anxiety, self-endangering behavior, and hypervigilance that are linked to maternal dysregulation in the context of maternal interpersonal violence exposure-related PTSD and aggression.
3) Decreased neural activity in maternal brain regions associated with emotion regulation (i.e. mPFC) following exposure to film scenes evoking escalation to male-female violence in comparison to a control condition that will in turn be related to an increase in child symptoms such as separation anxiety, self-endangering behavior, and hypervigilance.

2. Methods

2.1. Participants and procedures

The institutional ethics committee at the Geneva University Hospitals approved this research project which is in accordance with the Helsinki Declaration [34]. Participants gave written informed consent both for themselves as well as their child. Women and their young children were recruited by flyers posted at the Geneva University Hospitals and Faculty of Medicine and other

Faculties, as well as at community centers, daycares, pre-schools, and domestic violence agencies and shelters. Any mother and child dyad who responded and who followed through with an appointment was screened. Fathers and other partners of mothers were not seen in the study due to concerns over safety and maintenance of trust for women who had experienced partner violence. Exclusion criteria were as follows: Non-biological mothers, mothers who had not lived with their child for the majority of the child's life since birth, mothers who experienced symptoms of psychosis or active substance abuse or had mental or physical disability that would preclude participation in research tasks. Due to physiological measurements taken, women who were pregnant or breast-feeding were not accepted into the study. Children were included in the study if they were 12–42 months of age at the time of scheduled mother-child behavioral observations and if they had no mental or physical disability that would preclude participation in research tasks.

Within one month after the screening visit, participants completed two videotaped visits over the ensuing 1–2 month period. During the screening visit, following informed consent, mothers were given a socio-demographic and life-events interview followed by several self-report questionnaires. During the next visit, mothers were interviewed without their child present, with a focus on the mother's mental representations of her child and relationship with her child, an elaboration of her traumatic life-events, followed by structured diagnostic interviews and a series of dimensional measures. Then, 1–2 weeks later, mothers were asked to bring their child to the lab for a mother-child interaction procedure otherwise known as the "Modified Crowell Procedure" [35]. This procedure involves free play, separation-reunion, structured play, repeated separation-reunion and exposure to novelty. This mother-child interaction procedure was followed by administration of measures focusing on the child's life events, psychopathology, and social-emotional development. Saliva samples were taken for DNA extraction (as described in more detail below) prior to the Modified Crowell Procedure. After each of these visits, mothers received 50 Swiss francs along with a small book or toy for their child following the parent-child visit.

Mothers who consented and were eligible for MRI scanning were invited within 2–4 weeks after the mother-child visit, to the hospital-based neuroimaging center. After a clinician and neuroimaging specialist-guided orientation to the MRI scanner and scanning process, mothers participated in the fMRI protocol as described below [18].

For a subset of 39 mothers (mean age mothers 34.4 years, SD = 5.7 years, mean age children: 26.9 months, SD. 8.2 months), datasets including fMRI data and successful DNA extraction from saliva were available for 5HT3a. Three mothers were excluded due to motion in the MRI and one due to a non-IPV related PTSD. Seventeen of the remaining 35 participants were mothers without PTSD (controls), and 18 mothers were diagnosed with IPV-PTSD.

2.2. IPV and other traumatic life events

History of traumatic events throughout the mothers' lifetime was assessed via two measures: the Brief Physical and Sexual Abuse Questionnaire (BPSAQ) [36], and the Traumatic Life Events Questionnaire (TLEQ) [37]. To avoid redundancy, the authors did not repeat items exploring childhood traumatic events on the TLEQ that had already been probed on the BPSAQ, a more comprehensive measure for childhood events. Scoring of the BPSAQ was undertaken as described in a paper by Schechter and colleagues [38]. The number of lifetime violent events was the sum of the number of items endorsed on the BPSAQ related to childhood physical abuse, sexual abuse, and exposure to domestic violence added to the number of items endorsed on the TLEQ related to partner and non-partner physical and sexual assault during adulthood as well as military combat, other exposure to war or terrorism, or community violence since birth. For purposes of grouping, endorsement of any of these interpersonal violent items on the BPSAQ and/or TLEQ that met the "A"-criterion for PTSD according to the DSM-IV and associated with PTSD symptoms would identify the subject as having IPV-PTSD. Endorsement of medical/surgical/obstetrical events, vehicular or other life-threatening accidents, exposure to natural disaster, or sudden loss meeting the A-criterion for PTSD according to the DSM-IV and associated with PTSD symptoms would identify the subject as having "non-IPV-PTSD". The latter subjects were excluded from analyses due to a small number of subjects (n = 15). The severity of maternal aggression (i.e. use of verbal threats and physical violence) in the context of adult romantic relationships was measured via the Conflicts Tactics Scale-2, Short Version (CTS2) [39]. PTSD diagnosis was determined via the Clinician Administered PTSD Scale (CAPS) [40]. Mothers were included in the IPV-PTSD diagnosis group if they had experienced an interpersonal violent event that met the PTSD A-criterion and their CAPS score was greater than or equal to 55. Maternal depressive symptoms were assessed via the Beck Depression Inventory-II [41] as a self-report measure for the current subjective symptom severity.

Child psychopathology was measured via the Disturbances of Attachment Interview [42] which is a 12-item clinician-rated measure that takes into account maternal report as well as clinical observation and judgment. Of its three subscales: inhibited and disinhibited attachment and secure base distortion, we only included the latter subscale, as based on clinical indices from a prior study [43] One year after the initial evaluation, the Child Behavior Checklist for Children 1.5–5 Years [44] was mailed to mothers for completion and return. Of this measure, the aggressive behavior subscale only was included in analyses.

2.3. Saliva sampling and DNA extraction

Participants were instructed not to eat or drink for one hour prior to the test. Subsequently, a trained technician asked each participant to chew on a Salivette® swab for 3 min. The Salivette® swab was then placed in a labeled plastic tube and frozen at −30 °C. DNA was extracted with a specific extraction kit (GE Healthcare RPN 8501, Glattbrugg, Switzerland). We conducted quantification analysis of DNA samples with Qubit (the Qubit® 2.0 Fluorometer, Invitrogen) and the quality of DNA fragments was verified with gel electrophoresis. We then modified 2 μg of extracted DNA with sodium bisulfite via EpiTect Bisulfite Kit (Quiagen, Germantown, MD, USA) according to the manufacturer's protocol. PCR amplifications were performed using primers specific for each *5ht3aR* assays with the HotStarTaq Master Mix Kit (Qiagen, California, USA) on 2 μl of the post bisulfite-treated DNA. A vacuum workstation was used to isolate single stranded biotinylated DNA molecules in presence of the corresponding sequencing primers. Nucleotides, enzyme and substrate for pyrosequencing were from Qiagen (PyroMark Gold Reagents) and the reactions were performed on a PyroMark Q96 MD instrument. The degree of CpG methylation was measured in duplicates and automatically by the Pyro Q-CpG Software (Biotage AB, Uppsala, Sweden) by pyrosequencing. Given previous work [29] indicating a link between child maltreatment and modifications in the methylation status of CpG sites located in the promoter region of the *HTR3A*, seven *HTR3A* CpGs sites from Perroud et al. (2016) [29] were examined in the present study (CpG1_I, CpG2_II, CpG3_II, CpG2_III, CpG3_III, CpG4_III, CpG5_III) (Supplementary Fig. S1). In addition, previous work indicated that the methylation status of CpG2_III located in close vicinity of a functional SNP

(RS1062613) modulates binding of the transcription factor CTCF [45].

2.4. MRI procedure

Given the particular importance of intimate-partner violence among women with IPV-PTSD, we used video stimuli depicting neutral, menacing, and male-female prosocial interactions as described in a previous study that drew on the same sample [18]. Twenty-three silent 20-s video excerpts that displayed male-female interactions were extracted from feature films. These excerpts were grouped into 3 conditions: 8 excerpts displayed menace and high levels of negative affect, 8 displayed prosocial/romantic interaction and moderately to highly positive affect, and 7 displayed neutral affect. The categorization of those excerpts was done by an unpublished rating study, the details of which can be found in the Supplementary materials. Detailed image acquisition and pre-processing are described in the Supplementary materials. After the MRI visit, mothers received 200 Swiss francs.

2.5. Data analyses

All data were analyzed with SPSS version 22 (IBM Corp., Armonk, NY, USA). For behavioral data, statistical significance was fixed at $p < 0.05$. The sample was restricted to the 35 mothers who had complete data with respect to 5-HT3a methylation, MRI, and clinical measures. Correlations between *HTR3A* methylation, behavioral and questionnaire data were performed using the Spearman correlation coefficient, since a Kolmogorov-Smirnov test for normality of the data was significant across CpGs ($p \leq 0.05$). We, furthermore, performed posthoc tests for each of the brain activity clusters that we identified on fMRI analyses (as specified in the next paragraph). These posthoc tests correlated the mean activity of each identified cluster with maternal IPV-PTSD, and *HTR3A* methylation levels.

Specifically, in first level fMRI analysis, we produced a contrast between the average neural activity in response to seeing male-female interactions depicting menace as compared to scenes of romantic-prosocial and as compared to scenes of neutral interactions such as joint attention. Both comparisons were used to test whether effects were due to a response particular to menacing interactions rather than to the contrast conditions. In 2nd level analysis we applied correlations to examine the associations between this contrast and an ordinal scaled version of the mean *HTR3A* methylation (to account for non-normality of that data) within a whole-brain analysis. A cluster-extent based thresholding approach was used to correct for multiple comparisons created by the high number of voxels analyzed. A previous study had indicated that via simulation with 10,000 iterations, a false positive probability of 0.05 was achieved under the condition that each reported regional cluster include at least 27 contiguous voxels ($3\,mm \times 3\,mm \times 3\,mm$) with an uncorrected $p < 0.005$ [46]. For our whole-brain analysis, the threshold of significance was thus defined as an uncorrected $p < 0.005$ with at least 27 contiguous voxels necessary to constitute a significant finding. A cluster-extent based thresholding approach was used to correct for multiple comparisons: A Monte Carlo simulation with 10,000 iterations indicated that a false positive probability of 0.05 (i.e. 95% confidence) was achieved when implementing the condition that a cluster of at least 27 contiguous voxels displays an effect with $p < 0.005$. In order to limit further the potential for Type I error, testing of a-priori hypotheses were limited to two comparisons of neural activity and 1) the single CpG2_III of interest as well as 2) maternal IPV-PTSD severity. Whole brain correlations of maternal brain activity with CpGs other than CpG2_III can be found in the Supplementary materials and are only given for the sake of completeness.

3. Results

3.1. Characteristics of participants

Comparison of IPV-PTSD mothers (n = 18) and non-PTSD mothers (n = 17) via independent *t*-tests indicated no differences for maternal age, child age or gender. The socio-economic status (SES) of IPV-PTSD mothers was however significantly lower than non-PTSD controls (see Table 1). When we performed Spearman correlations looking at the association of maternal PTSD to 1) maternal experience of IPV in terms of number of violent life events on the BPSAQ and TLEQ and to 2) severity of adult partner violence (i.e. subscale score) on the CTS-2, while co-varying SES, these correlations remained highly significant. (see Supplementary Table S1)

We then examined group differences in terms of adverse life events exposure also using independent *t*-tests (see Fig. 1 and Supplementary Table S2.) As expected, the IPV-PTSD group of mothers has significantly higher rates of physical and sexual abuse and exposure to domestic violence as children, as well as exposure to physical and/or sexual violence as adults. The majority of mothers with adult exposure were physically abused (61%, likelihood ratio = 11.36, $p \leq 0.05$).

Differences between groups were significant in terms of the number of violent events experienced across the mothers' lifetime, and by definition, maternal IPV-PTSD, but also major depressive symptom severity. There was no significant group difference, however, for maternal aggressive behavior (see Table 2a). Non-parametric (Spearman) correlations showing similarly significant relationships between the number of violent life events across the mothers' lifetime and related PTSD with severity of maternal depressive symptoms, maternal aggression and child SBD are shown in the Supplementary materials (See Supplementary Table S3).

Comparison of IPV-PTSD and non-PTSD mothers with respect to continuous child psychopathology measures yielded significant results. Maternal IPV-PTSD was associated with the Secure Base Distortion subscale and in particular, three component behaviors, self-endangering behavior, separation anxiety, and hypervigilance on the Disturbances of Attachment Interview (DAI) when children were ages 12–42 months (Time 1).

3.2. HTR3A *gene methylation at CpG2_III*

We then examined group differences by independent *t*-tests for methylation of the seven 5-*HTR3A* CpGs studied (see Table 2b). IPV-PTSD mothers were characterized by a significantly lower mean percentage of methylation of the *HTR3A* gene promoter region at two of the CpG sites in the promoter region: 2_III and 3_III and a significantly higher mean percentage of methylation in the two CpGs located in the coding region: 4_III and 5_III.

Based on our first and second a-priori hypotheses relative to methylation in the gene's promoter region (see Section 1.4), we performed comparisons using Spearman correlation of 5 key measures (see Table 3): number of violent events experienced by mothers in their lifetime from the TLEQ, maternal PTSD severity from the CAPS, maternal major depression symptom severity on the Beck Depression Inventory II (BDI-II), maternal aggression on the CTS-2, and the child attachment disturbance "secure base distortion" on the DAI, with its 4 component behaviors. Of note, *HTR3A* methylation at CpGs 2_III and 3_III was significantly associated after Bonferroni testing with maternal lifetime violence exposure, lifetime IPV-PTSD severity, maternal aggression and child self-endangering behavior on the DAI.

We additionally looked post-hoc specifically at the relationships of child physical and sexual abuse on the BPSAQ with methylation

Table 1
Group differences in socio-demographic variables.

	IPV-PTSD (n = 18)	Controls (n = 17)	t (35 d.f.)/pearson chi-square	P
Maternal age (in years)	33.53 (5.76)	35.59 (5.54)	1.120 (2,33)	0.27
Child age (in months)	26.78 (8.34)	27.12 (8.34)	.33 (2,33)	0.95
% Boys	55%	53%	.024 (1,34)	0.88
Socio-economic status (low values indicate higher status)	5.82 (2.01)	4.35 (1.94)	−2.18 (2,33)	0.04

This table shows no significant group differences between IPV-PTSD mothers and children versus non-PTSD controls with respect to key socio-demographic variables.

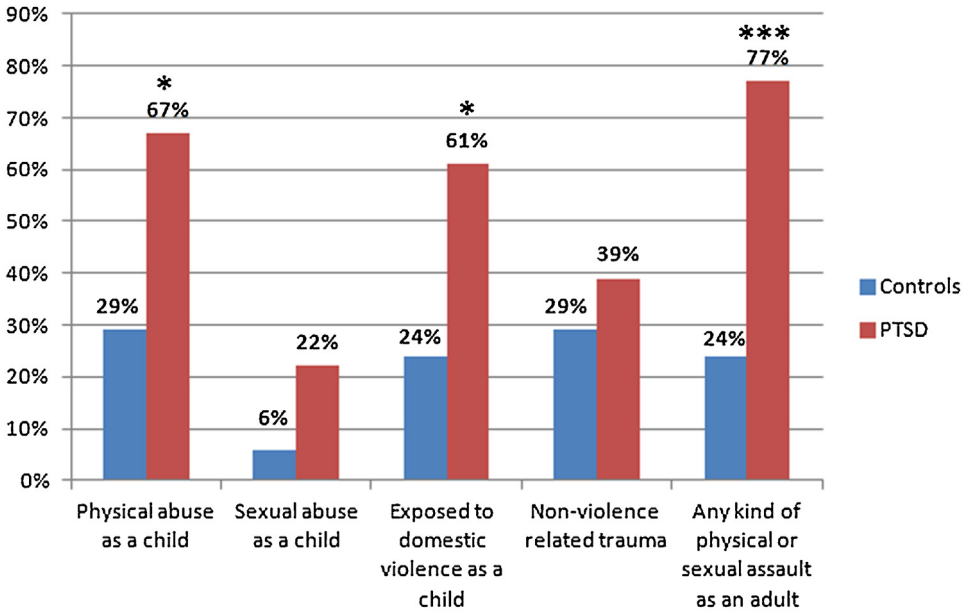

Fig. 1. Group differences in the distribution of maternal traumatic life events (N = 35).
This figure shows group differences in the distribution of traumatic life events between IPV-PTSD mothers in red and non-PTSD control mothers in blue. Maternal exposure to child physical abuse, domestic violence exposure, and any kind of physical or sexual violence as an adult showed a significant group difference (*p < 0.05; **p < 0.01, ***p < 0.005).

Table 2a
Group differences in maternal and child trauma and psychopathology variables.

	IPV-PTSD (n = 18)	Controls (n = 17)	t (d.f. 2,33)	p
Maternal variables				
Number of violent events (lifetime)	3.61 (4.02)	0.24 (0.44)	−3.54	0.002
Maternal PTSD lifetime severity (CAPS)	80.56 (20.92)	21.65 (14.67)	−9.59	0.000
Maternal depression (BDI)	10.50 (8.45)	3.82 (3.17)	−3.06	0.004
Maternal aggression (CTS)	4.19 (4.79)	2.21 (1.94)	−1.59	0.121
Child variables				
Child attachment disturbance: Secure Base Distortion (DAI) at 12–42 mos	2.89 (1.97)	1.00 (1.41)	−3.18	0.003
Child self-endangering behavior (DAI) at 12–42 mos	0.94 (0.73)	0.38 (0.50)	−2.63	0.013
Child separation anxiety (DAI) at 12–42 mos	0.78 (0.73)	0.31 (0.60)	−2.01	0.053
Child hypervigilance (DAI) at 12–42 mos	0.72 (0.83)	0.13 (0.05)	−2.58	0.015
Child role-reversal (DAI) at 12–42 mos	0.47 (0.72)	0.27 (0.59)	−0.87	0.390

This table shows a number of significant group differences between IPV-PTSD mothers and children versus non-PTSD control mothers and children with respect to trauma history and psychopathology variables. Those *t*-tests which remained significant following Bonferroni correction are shown in bold-face type.

Table 2b
Group differences in methylation of serotonin receptor 3A.

Maternal *HTR3A* methylation	IPV-PTSD (n = 18)	Controls (n = 17)	t (d.f. 2,33)	p
CPG1-I	60.51 (9.27)	65.79 (10.71)	1.56	0.128
CpG1-II	86.38 (15.70)	93.81 (11.62)	1.59	0.122
CpG2-II	33.42 (11.68)	32.44 (16.01	−0.21	0.836
CpG2-III	83.08 (11.48)	93.23 (10.14)	2.77	0.009
CpG3-III	85.88 (11.50)	93.75 (9.56)	2.20	0.035
CpG4-III	84.15 (12.35)	65.90 (19.67)	−3.31	0.002
CpG5-III	64.29 (9.40)	50.01 (17.05)	−3.09	0.004

This table shows significant group differences between mean percent methylation across the 7 CpG sites tested between IPV-PTSD mothers and non-PTSD controls. Those *t*-tests which remained significant following Bonferroni correction are shown in bold-face type.

Table 3
Correlation matrix for the 5 key mother-child trauma and psychopathology variables.

		CPG1_I	CpG1_II	CpG2_II	CpG2_III	CpG3_III	CpG4_III	CpG5_III
	Maternal variables							
1.	Number of violent events (TLEQ-CAPS)	−0.36*	−0.23	0.11	−0.42**	**−0.43****	0.32*	0.42**
2.	Maternal PTSD lifetime severity (CAPS)	−0.35*	**−0.51*****	0.01	**−0.51*****	−0.41**	0.37*	0.35*
3.	Maternal depression (BDI)	−0.35*	−0.45**	0.09	−0.30	−0.27	0.23	0.25
4.	Maternal aggression (CTS)	−0.13	−0.20	0.21	−0.39*	**−0.43****	0.03	0.13
	Child variables							
5.	Child attachment disturbance: Secure Base Distortion Subscale (DAI)	−0.12	−0.24	0.24	−0.38*	−0.35*	**0.44****	**0.48****
5a.	Child self-endangering behavior item of Secure Base Distortion Subscale (DAI)	−0.31	−0.33	−0.03	**−0.46*****	**−0.44****	**0.49****	**0.61*****
5b.	Child separation anxiety item of Secure Base Distortion Subscale (DAI)	−0.09	−0.38*	0.13	−0.31	−0.31	0.27	0.33
5c.	Child hypervigilance item of Secure Base Distortion Subscale (DAI)	−0.10	−0.28	−0.32	−0.17	−0.16	0.21	0.29
5d.	Child role-reversal item of Secure Base Distortion Subscale (DAI)	−0.27	0.14	0.17	−0.15	−0.10	0.01	0.11

This table shows Spearman (non-parametric correlations) that were performed across the entire sample of the 5 key mother-child trauma and psychopathology variables drawn from the study's a-priori hypotheses. Those correlations which remained significant following Bonferroni correction are shown in bold-face type.
* $p \leq 0.05$.
** $p \leq 0.01$.
*** $p \leq 0.005$.

at the CpG 2_III and 3_III sites. These associations failed to reach significance for CpG 2_III and physical abuse r = −0.15, p > 0.4; for CpG 2_III and sexual abuse r = −0.27, p =0.14; for CpG 3_III and physical abuse r = −0.13, p > 0.4; and for CpG 3_III sexual abuse r = −0.19, p > 0.3. However, these two CpG's were significantly associated with adult physical and sexual assault on the BPSAQ (for CpG 2_III and adult abuse r = −0.42, p = 0.01; for CpG 3_III, r = −0.40, p = 0.02) and severity of self-rated partner violence on the CTS2 (for CpG 2_III and partner violence r = −0.41, p = 0.02; for CpG 3_III, r = −0.44, p = 0.008).

Out of 35 subject-mothers, 30 completed the Child Behavior Checklist (CBCL) one-year later. On post-hoc analysis, the CBCL aggressive behavior subscale score correlated with the DAI self-endangering behavior item (r = 0.42, p = 0.02). Child aggression on the CBCL and the other three items of the DAI Secure Base Distortion subscale (i.e. separation anxiety, hypervigilance, and role reversal) were not significantly correlated (p values respectively = 0.12, 0.82, 0.45).

3.3. *fMRI results*

In order to test our hypothesis that less neural activity would be found in regulatory areas of the maternal brain upon viewing of stimuli evocative of IPV, as associated with *HTR3A* methylation at the CpG 2_III site, we examined the relationship of percentage of methylation of the *HTR3A* gene promoter region at the CpG 2_III to maternal neural activity in response to mothers' viewing of menacing vs prosocial as well as menacing vs neutral interactions between adult men and women as drawn from fiction films (see Fig. 2 and Table 4). The percentage of methylation of the *HTR3A* gene promoter region at the CpG 2_III site significantly correlated with activity in the dorsomedial prefrontal cortex (dmPFC), and left dorsolateral prefrontal cortex (dlPFC) regardless of whether menacing male-female interactions were paired with prosocial or neutral control stimuli conditions. The relationship of the percentage of methylation of the *HTR3A* gene promoter region at the other 6 CpG sites are shown in Supplementary materials (see Supplementary Tables S4–S9).

3.4. *Regression model*

Given our third *a priori* hypothesis that mPFC dysregulation plays an important role in PTSD patients' response to traumatic triggers and, in turn, that mPFC dysregulation would relate both to *HTR3A* methylation at the CpG 2_III site, and to child self-endangering behavior given these two variables' significant association to each other, we entered a) region of interest activity

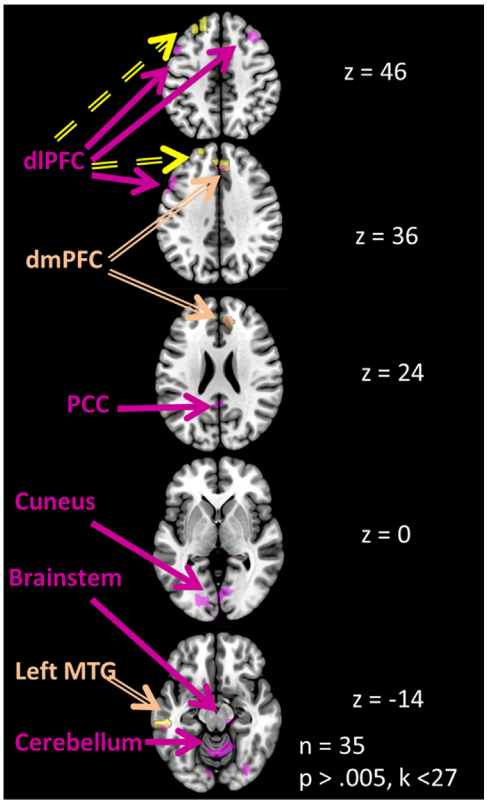

Fig. 2. Correlation of maternal *HTR3A* methylation at CPG 2_III site and maternal neural activity in response to film excerpts of adult men and women in both menacing versus neutral and menacing versus prosocial interactions.
This figure shows correlations of maternal *HTR3A* methylation at CPG 2_III site and maternal neural activity in response to film excerpts of adult men and women in both menacing versus neutral and menacing versus prosocial interactions in order to show that exposure to the menacing stimuli is associated with decreased dmPFC and left medial-temporal gyrus activity in both comparisons. This is represented by the hollow salmon colored arrows, which shows the overlap of effects in both menacing vs neutral and menacing vs prosocial comparisons. Negative correlations between *HTR3A* methylation at CPG 2_III site and maternal neural activity in response to menacing vs neutral interactions between adult men and women are represented by hollow intermittent yellow arrows. Negative correlations between *HTR3A* methylation at CPG 2_III and maternal neural activity in response to menacing vs prosocial interactions between adult men and women are represented by magenta-colored arrows.
Abbreviations: dmPFC, dorsal medial prefrontal cortex; dlPFC, dorsal lateral prefrontal cortex; PCC, posterior cingulate cortex; MTG, middle temporal gyrus.

Table 4
Maternal neural activity in response to menacing versus prosocial adult male-female interactions in relation to methylation at the 5HT3a CpG 2_III site.

cluster size	MNI location of the peak voxel			Regions comprised in this cluster	Peak voxel		Correlation of cluster activity with *5ht3ra* rank		Correlation of cluster activation with rank of 5ht3a mean methylation	Correlation of cluster with severity of IPV-PTSD
	x	y	z		t	p	r value within: IPV-PTSD HC	p value within: IPV-PTSD HC		
Negative correlation with menacing vs prosocial										
50	9	41	25	Dorsomedial Prefrontal Cortex, dorsal anterior Cingulate Cortex	3.40	0.001	−0.180 −0.401	0.475 0.110	r = −0.518 p = 0.001	r = 0.430 p = 0.010
88	−54	14	34	Left Dorsolateral Prefrontal Gyrus, Left Precentral Gyrus	4.28	<0.001	−0.288 −0.425	0.246 0.089	r = −0.603 p < 0.001	r = 0.393 p = 0.020
44	36	26	46	Right Dorsolateral Prefrontal Gyrus	3.76	<0.001	−0.295 −0.698	0.234 0.002	r = −0.538 p = 0.001	r = 0.394 p = 0.019
72	−33	11	−29	Left Temporal Pole, Left Superior Temporal Gyrus	4.22	<0.001	−0.244 −0.529	0.330 0.029	r = −0.608 p < 0.001	r = 0.387 p = 0.022
28	−57	−31	−14	Left Middle Temporal Gyrus	4.00	<0.001	−0.076 −0.609	0.765 0.009	r = −0.563 p < 0.001	r = 0.264 p = 0.126
41	0	−49	19	Posterior Cingulate Cortex	3.75	<0.001	−0.386 −0.364	0.114 0.150	r = −0.539 p = 0.001	r = 0.511 p = 0.002
329	−21	−88	1	Left cuneus, V1	4.28	<0.001	−0.119 −0.575	0.639 0.016	r = −0.544 p = 0.001	r = 0.325 p = 0.057
142	6	−58	−20	Cerebellum	3.35	0.001	−0.496 −0.455	0.036 0.066	r = −0.501 p = 0.002	r = 0.298 p = 0.082
90	30	−85	−26	Cerebellum	3.96	<0.001	−0.416 −0.404	0.086 0.108	r = −0.625 p < 0.001	r = 0.428 p = 0.010
70	12	−28	−8	Right Brainstem	3.47	0.001	−0.311 −0.475	0.209 0.065	r = −0.571 p < 0.001	r = 0.286 p = 0.095
Negative correlation with menacing vs neutral										
69	12	41	25	dmPFC	3.55	0.001	−0.108 −0.411	0.669 0.101	r = −0.503 p = 0.002	r = 0.472 p = 0.004
109	−21	38	49	Left dlPFC	4.09	<0.001	−0.055 −0.462	0.829 0.062	r = −0.565 p< 0.001	r = 0.535 p = 0.001
111	−33	14	−26	Left Temporal Pole	4.47	<0.001	−0.620 −0.340	0.006 0.181	r = −0.610 p< 0.001	r = 0.374 p = 0.027
34	−57	−28	−17	Left Middle Temporal Gyrus	3.75	<0.001	−0.055 −0.551	0.143 0.022	r = −0.502 p = 0.002	r = 0.378 p = 0.025

This table shows significant maternal activity of brain regions in response to silent film clips of menacing versus prosocial and menacing versus neutral adult male-female interactions on fMRI correlated to the methylation of the *HTR3A* CpG 2_III site. For inclusion, the correlation between neural activity and methylation of this HTR3A CpG had to be significant in at least 27 contiguous voxels of $p < 0.005$ each. Additional columns were included to allow understanding of the origin of effects.
Abbreviations: dmPFC, dorsomedial Prefrontal Cortex; dlPFC, dorsolateral Prefrontal Cortex; OFC, Orbitofrontal Cortex; vmPFC, ventromedial Prefrontal Cortex; V1, primary visual cortex.

of mothers' dmPFC in response to menacing vs. prosocial scenes, into a regression model with b) mean percentage of methylation of mothers' *HTR3A* gene promoter region at the *CpG* 2_III as independent predictors of child self-endangering behavior (DAI). The dmPFC region of interest was taken from the anatomic automatic labelling databases' definition of the bilateral medial frontal gyrus. We found a significant combined model explaining 26% of the variance: F = 5.75 (df 2,32), p = 0.007, β-methylation of *HTR3A* CpG 2_III = −0.28, t = −1.76, p = 0.09; β-dmPFC activity 0.36, t = −2.27, p = 0.03. β-Methylation of *HTR3A* CpG 2_III alone = −0.38, p = 0.024 explaining 14% of the variance; and β-dmPFC activity alone = 0.43, p = 0.009 explaining 19% of the variance. Controlling for maternal socio-economic status (SES) did not significantly alter the regression model (β-SES 0.003, t = 0.016, p = 0.99).

4. Discussion

4.1. Convergent associations between maternal 5HT3aR methylation, trauma history, psychopathology, and neural activity

Results of this study have shown significant and convergent associations between the mean percentage of methylation of the promoter region of the *HTR3A* gene in salivary DNA and the following key variables: maternal history of exposure to childhood maltreatment and subsequent violent victimization, related IPV-PTSD, maternal aggressive behavior and child attachment disturbance of the secure base distortion type and in particular one of its cardinal signs, self-endangering behavior. The peripheral level of *HTR3A* methylation despite its unknown relationship to central nervous system levels of methylation, may thus represent a marker for a maternal IPV-PTSD endophenotype that confers risk to the mother-child relationship and the child's social-emotional development. These converging results were most significantly found to involve less methylation at one particular CpG site in the *HTR3A* gene's promoter region CpG 2_III which was identified as being associated with child maltreatment in a previous study by Perroud et al. that also used peripheral DNA (i.e. blood) [29]. The fact that this CpG site is located only 1 base-pair distance from a functional single-nucleotide polymorphism (SNP) RS1062613, which has been previously linked to early adverse experience [25] provides insights into how genetic and epigenetic factors may interact to regulate *HTR3A* expression. In addition, in vitro gel shift assays indicate that allele-specific methylation of CpG2_III increases the binding of the transcription factor CTFC to the promoter region of *HTR3A* [45]. Furthermore, in silico molecular dynamic simulation shows that binding of CTCF to the methylated CpG2_III site of the *HTR3A* allele is stronger and more stable compared to the un-methylated condition [45]. At a functional level, increased CTCF binding to *HTR3A* has been suggested to repress transcription [45]. These results suggest that in PTSD, de-methylation of CpG2_III may decrease binding of CTCF to the *HTR3A* allele and possibly increase transcription. Although the functional consequences of CTCF binding to HT3AR remain to be clearly established, the present study further points to the clinical importance of CpG2_III, by showing a significant association between methylation at CpG 2_III and maternal IPV-PTSD. The specificity of this association is supported by the observation that *HTR3A* methylation at CpG 2_III was not significantly associated with maternal major depressive symptoms.

The above described convergent associations implicating the CpG 2_III site were also found to be linked to maternal neural activity in cortical brain regions involved in emotion and arousal regulation. Interestingly, *HTR3A* methylation at CpG 2_III was associated to neural activity in the dmPFC in response to silent video stimuli depicting adult men behaving in a physically menacing manner towards adult women as compared to a prosocial or emotionally neutral manner. These neuroimaging findings extend our previous studies, in which dmPFC as well as vmPFC activity in response to this same silent video paradigm were found to be negatively and significantly associated with maternal IPV-PTSD severity [18]. Moreover, neural activity in these same mPFC regions in response to parenting specific stimuli (i.e. silent video clips of own and unfamiliar toddlers in separation versus play) have also been found to be significantly associated both to peripheral measures of glucocorticoid receptor gene *NR3C1* promoter region and to maternal IPV-PTSD severity [31]. Additionally, dlPFC and vmPFC activity have been associated both to observed maternal sensitivity [47] and maternal methylation of the *BDNF* gene promoter region [48]. Overall, The present paper further suggests that the combination of peripheral *HTR3A* gene methylation signatures that are associated with relevant functional brain activity in key cortico-limbic regions is a useful approach to begin delineating the maternal endophenotypes of IPV-PTSD as distinct from other forms of psychopathology.

Indeed, some important differences from the Perroud et al. [29] study, which are related to this distinction, are worthy of mention. Firstly, Perroud's study did not consider PTSD or neural activity. Secondly, while methylation of the promoter region of the *HTR3A* gene was negatively associated with the number of interpersonal violent (IPV) events to which the mother had been exposed since childhood- and on post-hoc analyses, the presence and severity of adult physical and sexual assault including domestic violence to which most of the mothers with PTSD in this particular sample were exposed, this gene was not specifically associated with child physical abuse as it had been in that previous study [29]. That being said, 60% of the mothers who had experienced interpersonal violence as adults had experienced physical abuse as children. Although, an association with child sexual abuse fell short of significance (p = 0.14). Thus, negative findings with respect to child physical and sexual abuse may be a result of the limited sample size.

Furthermore, whereas Perroud's study [29] found increased methylation to be associated with child maltreatment, greater severity of suicide attempts, number of hospitalizations, and mood disorder episodes, the present study found that decreased methylation was associated with maternal history of violent trauma, related PTSD diagnosis and severity, relational aggression, and less activation in the dmPFC. The explanation for these differences may lie in the fundamental difference in the pathophysiology of PTSD versus major depressive disorder [32,33], which we similarly asserted was responsible for the difference of direction of methylation effect in the instance of the relationship of *NR3C1* and its associations with maternal IPV-PTSD, parenting stress, and neural activity in the mPFC [31]. Nevertheless, these are but two studies that require replication with larger samples that include subjects suffering from PTSD, mood disorder, and their comorbidity.

4.2. Interpreting the link of maternal findings to child attachment disturbance and self-endangering behavior

Methylation of the *HTR3A* receptor promoter region, particularly at the functional site CpG2_III was negatively correlated with maternal verbal and physical aggression as measured on the Conflict Tactics Scale-2 (CTS2), a measure which looks at how interpersonal conflicts are treated in adult romantic relationships. In the context of IPV, maternal caregiving is more unpredictable, with less availability to the child for mutual emotion regulation of emotion and arousal during sensitive periods of emotional development [43].

Interestingly, on a clinician rated measure, the DAI, that employs child-parent observation, child attachment disturbance in the form of Secure Base Distortion (SBD) was linked to less methylation of the *HTR3A* promoter region. Maternal IPV-PTSD has been indeed

directly associated with SBD in a prior study [43]. SBD consists of four symptoms that clinicians can rate on the DAI [49]: namely, separation anxiety, self-endangering behavior, hypervigilance and role reversal [43]. We noted in this study that the self-endangering behavior item individually, while not significantly associated with maternal PTSD, was strongly and negatively correlated with less methylation of the maternal *HTR3A* particularly at CpG 2_III and CpG 3_III (see Table 4 and Supplementary Table S7). And in turn, we found that self-endangering behavior was strongly associated with child aggressive behavior on a standard parental report measure one year later for the 30 out of the 35 children whose mother's completed the questionnaire. Together, two indicators of reduced maternal emotional regulation capacities: namely, methylation of the maternal *HTR3A* receptor at CpGs 2_III and reduced maternal dmPFC activation, accounted for nearly one-third of the variance of child self-endangering behavior in the applied multiple regression analysis. It might be the case that in the absence of a predictable primary attachment figure and role-model for mutual emotion regulation during sensitive periods of early social-emotional development, the toddler can develop an attachment disturbance that is characterized by emotional dysregulation, impulse dyscontrol, and self-endangering behavior. Further study that would include child *HTR3A* methylation is needed to clarify whether the epigenetic signature of *HTR3A* as observed in this sample might be a marker of a more aggressive and self-endangering child endophenotype in this context.

4.3. Limitations

This study was clearly limited by a small sample size especially given 1) limited amount of good quality salivary DNA for pyrosequencing 2) constraining the sample only to mothers who had a) consented to participate in MRI scanning and b) who had complete and adequate MRI data. Moreover, child salivary DNA was not available for pyrosequencing. Another clear limitation is the use of peripheral salivary DNA from which methylation levels in brain cells cannot be inferred. And thus, in this paper, the authors have restricted their interpretation of the findings to suggest a possible peripheral biomarker of a maternal endophenotype characterized by heightened aggression in the context of maternal IPV-PTSD. The data do not permit any causal inference concerning the relationship between maternal peripheral *HTR3A* methylation and neural activity or between either of these two variables and maternal-child symptoms and behavior. In the rodent brain, the HTR3AR has been shown to be specifically expressed in a subset of inhibitory GABAergic interneurons located in a variety of brain regions, including the mPFC, hippocampus and amygdala [20,21,23]. Whether early-life adversity affects the methylation status of the *HTR3A* receptor in this subset of GABAergic interneurons remains to be tested in rodent models of early-life stress. Furthermore, the impact of stress-induced *HTR3A* methylation changes on the expression levels of the *HTR3A* transcript remains to be determined. In a broader perspective and in relationship with PTSD, the *HTR3A* receptor has been shown to be required for fear extinction. Indeed, mice with a constitutive genetic deletion of the *HTR3A* receptor fail to normally extinguish conditioned fear responses [24]. Given the critical role of the mPFC in controlling fear extinction [50], the precise circuit mechanisms through which the *HTR3A* receptor regulates fear extinction in the mPFC remain to be established. Overall, our data indicate that mPFC neural activity and peripheral blood *HTR3A* receptor methylation status are linked. However, much work remains to be done to understand the role of the *HTR3A* receptor in mPFC microcircuit function in PTSD-related models.

The use of retrospective measures to evaluate childhood abuse and violence exposure history is another potential limitation. Recent studies on potential retrospective reporting bias concerning childhood adverse experiences have found, however, that retrospective reports tend to be at least moderately reliable when reliable life-events measures are used as was the case in the present study [51,52]. A further limitation was the absence of a clinician-rated child diagnostic measure apart from the DAI to look at additional psychiatric diagnoses and, in particular aggressive behavior at the time of the initial evaluations and then again, one year later. Finally, while several important associations remained significant even after correction for multiple comparisons (i.e. *HTR3A* methylation being inversely related both to maternal IPV-PTSD diagnosis and symptom severity as well as to child self-endangering behavior on the DAI and to decreased neural activity in the maternal dmPFC in response to relational stimuli), given the small sample size, several other associations related to our a-priori hypotheses did not survive the Bonferroni correction. This again suggests the need for replication of this study within a larger sample.

4.4. Conclusions

In conclusion, this paper supports the notion that violent trauma beginning in infancy and early childhood leaves an epigenetic signature on a serotonin receptor gene that has been shown to be implicated in the regulation of emotion and aggression, namely, *HTR3A*. This peripheral epigenetic signature among mothers of very young children corresponds to a number of maternal psychopathology, behavior, and neural activity measures that, similarly, are implicit to the regulation of emotion and aggression. And as such, the significant association of *HTR3A* methylation with the child attachment disturbance SBD, as marked by child self-endangering behavior is particularly intriguing. Further research is needed to understand if methylation of the *HTR3A* gene promoter region particularly at the functional CpG 2_III site reflects a potentially aggressive, impulse-disinhibited (versus anxious, impulse-inhibited) maternal endophenotype that might impact the intergenerational transmission of violent trauma.

Acknowledgements

This research was supported by the National Center of Competence in Research (NCCR) "SYNAPSY – The Synaptic Bases of Mental Diseases" financed by the Swiss National Science Foundation (n° 51AU40_125759), the Gertrude von Meissner Foundation, and la Fondation Prim'Enfance, Switzerland.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2016.10.009.

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